

AMENDMENTS TO THE SPECIFICATION

Please amend the specification, as follows:

Please insert the following header and paragraph at page 1 immediately following the Title of the Invention:

CROSS-REFERENCE TO RELATED APPLICATION

This application is a national stage of International Application No. PCT/JPO3/06841, filed May 30, 2003.

Please replace the paragraph at page 3, line 18, with the following amended paragraph:

~~Fig. 1 shows~~ Figs. 1A-1H show the results of an in vitro binding experiment using PEG-Chol.

Please replace the paragraph at page 3, lines 19-20, with the following amended paragraph:

~~Fig. 2 shows~~ Figs. 2A-2R show the results of a labeling experiment with PEG-Chol using cells. The bar indicates 20 rim.

Please replace the paragraph at page 3, lines 21-22, with the following amended paragraph:

~~Fig. 3 shows~~ Figs. 3A-3F show the results obtained by examining the distribution of fPEG-Chol on the surface of cells.

Please replace the paragraph at page 3, lines 23-24, with the following amended paragraph:

~~Fig. 4 shows~~ Figs. 3G-3L show the results obtained by examining the distribution of fPEG-Chol on the surface of cells.

Please replace the paragraph at page 3, lines 25-26, with the following amended paragraph:

~~Fig. 5 shows~~ Figs. 3M-3T show the results obtained by examining the distribution of fPEG-Chol on the surface of cells.

Please replace the paragraph at page 4, lines 1-2, with the following amended paragraph:

~~Fig. 6 shows~~ Figs. 4A, 4B, 4A' and 4B' show the results obtained by analyzing the intra-membrane distribution of cholesterol and the fate of cholesterol on the surface of cells.

Please replace the paragraph at page 4, lines 3-4, with the following amended paragraph:

~~Fig. 7 shows~~ Figs. 4C-6N show the results obtained by analyzing the intra-membrane distribution of cholesterol and the fate of cholesterol on the surface of cells.

Please replace the paragraph at page 3, line 18, with the following amended paragraph:

~~Fig. 8 shows~~ Figs. 40-4T show the results obtained by analyzing the intra-membrane distribution of cholesterol and the fate of cholesterol on the surface of cells.

Please replace the paragraph at page 14, lines 11-22, with the following amended paragraph:

In Figs. ~~4G to 4L~~ 3G to 3L, normal skin fibroblasts were labeled with 2 μ M fPEG-Chol. Thereafter, the cells were incubated with a 5 μ g/ml biotinylated epidermal growth factor (EGF) at 4°C for 20 minutes (Figs. ~~4G 3G~~ and ~~4H 3H~~), or at 37°C for 2 minutes (Figs. ~~[[4I and 4L]]~~ 3I and 3L). Thereafter, the cells were fixed with PBS containing 3% PFA and 8% sucrose, quenched, and then incubated with TRITC-labeled streptavidin at 4°C for 20 minutes. The specimens were observed with a Nikon TE 300 microscope equipped with a Hamamatsu C-4742-98 cooled CCD camera. In Fig. ~~[[4]]~~ 3, G and I indicate fPEG-Chol fluorescence, and H and J indicate AlexaFluor 594 EGF-fluorescence. In K and L in Fig. ~~[[4]]~~ 3, the cells were doubly labeled with 1 μ M fPEG-Chol and an AlexaFluor 594-labeled cholera toxin B subunit prior to being stimulated by non-labeled EGF. In Fig. ~~[[4]]~~ 3, K indicates fPEG-Chol fluorescence, and L indicates cholera toxin fluorescence. In Fig. ~~[[4]]~~ 3, the bar indicates 4 μ m.

Please replace the paragraph at pages 14, line 23 to page 15, line 4, with the following amended paragraph:

In M to P in Fig. ~~[[5]]~~ 3, B cell line A20.2J was incubated at 37°C for 1 minute without antibodies. Cells were then washed and fixed with 1% PFA for 30 minutes, and then labeled with 0.7 μ M fPEG-Chol and a 10 μ g/ml Alexa 546-conjugated cholera toxin B subunit in 0.1% BSA on ice for 45 minutes. After washing, the stained cells were observed under a Zeiss LSM 510 confocal microscope. In Fig. ~~[[5]]~~ 3, M indicates fPEG-Chol labeling, N indicates cholera toxin labeling, O indicates a merged image, and P

indicates a phase contrast image. Under these conditions, fPEG-Chol permeates the fixed cells, so as to stain intracellular membranes as well as plasma membranes. In contrast, cholera toxin did not enter the cells, and thus, it stained only the cell surfaces.

Please replace the paragraph at page 15, lines 5-9, with the following amended paragraph:

In Q to T in Fig. ~~[[5]]~~ 3, A20.2J cells were stimulated with 15 µg/ml F(ab')₂ goat antibodies specific for mouse IgG+IgM (F(ab')₂ anti-Ig) at 37°C for 1 minute. Thereafter, the cells were fixed and stained as described above. In Fig. ~~[[5]]~~ 3, Q indicates fPEG-Chol labeling, R indicates cholera toxin labeling, S indicates a merged image, and T indicates a phase contrast image.

Please replace the paragraph at page 15, lines 11-12, with the following amended paragraph:

In Example 3, the distribution of fPEG-Chol on the cell surface was examined (Figs. ~~3 to 5~~ 3A-3T).

Please replace the paragraph at page 15, line 23 to page 16, line 9, with the following amended paragraph:

Subsequently, the distribution of fPEG-Chol when cells were not stimulated with an epidermal growth factor (EGF) was measured. An EGF receptor localized to cholesterol-rich plasma membrane domains, and thus, it was suggested that the binding of EGF to the EGF receptor is dependent on cell surface cholesterol (M. G. Waugh et al.,

Biochem Soc Trans 29, 509-11. (2001); K. Roepstorff et al., J Biol Chem 277, 8 (2002); and T. Ringerike et al., J Cell Sci 115, 1331-40. (2002)). fPEG-Chol fluorescence was co-localized with the distribution of biotin-labeled EGF, when EGF was added at 4°C (Figs. ~~4G and 4H~~ 3G and 3H). When EGF was added at 37°C, the clustering of EGF receptors was observed (Fig. ~~[[4J]]~~ 3J). These clusters were labeled with fPEG-Chol (Fig. ~~[[4I]]~~ 3I). The cell surface distribution of GM1 was also examined under these conditions. GM1 was also enriched in these clusters and further co-localized with fPEG-Chol (Figs. ~~4K and 4L~~ 3K and 3L). These results indicate that EGF induces re-distribution of both cholesterol and GM1 to the same clusters where EGF receptors were enriched.

Please replace the paragraph at page 16, lines 10-18, with the following amended paragraph:

Re-distribution of plasma membrane ganglioside occurs during the cross-linking of B cell antigen receptors on the plasma membrane of a B cell line A20.2J (M. J. Aman et al., J Biol Chem 276, 46371-8. (2001)). Whether or not fPEG-Chol is re-distributed by treatment with F(ab')₂ anti Ig was examined. Before the treatment, both AlexaFluor 594-labeled cholera toxin and fPEG-Chol outlined the entire surface (Figs. ~~5M to 5P~~ 3M to 3P). However, after stimulation with a F(ab')₂ fragment for 1 minute, cholera toxin was accumulated in aggregated structures on the plasma membranes (Fig. ~~[[5R]]~~ 3R fPEG-Chol also localized to these structures (Figs. ~~5Q and 5S~~ 5Q and 5S). These results indicate that cholesterol is re-distributed together with GM 1 during stimulation of B cell lines.

Please replace the paragraph at page 16, lines 22-26, with the following amended paragraph:

(1) As described above, the plasma membranes of normal (~~Fig. 6A~~ Fig. 4A and 4A') and NPC (~~Fig. 6B~~ 4B) fibroblasts were permeabilized using streptolysin 0. The cells were incubated with fPEG-Chol at room temperature for 30 minutes before washing and taking fluorescence images under a Zeiss LSM 510 confocal microscope. The results are shown in ~~Fig. 6~~ Figs. 4A, 4A', 4B and 4B'.

Please replace the paragraph at page 17, lines 1-5, with the following amended paragraph:

(2) Normal (~~Figs. 7C to 7H~~ 4C to 4H) and NPC (~~Figs. 7I to 7N~~ 4I to 4N) fibroblasts were incubated with 1 μ M fPEG-Chol at room temperature for 5 minutes. Cells were washed and incubated for 10 minutes (Fig. ~~[[7]]~~ 4, F, L and L), 60 minutes (Fig. ~~[[7]]~~ 4, D, G, J, and M), and 180 minutes (Fig. ~~[[7]]~~ 4, E, H, K, and N) at 37°C in the presence of 1 mg/ml rhodamine dextran. The results are shown in ~~Fig. 7~~ Figs. 4C-4N.

Please replace the paragraph at page 17, lines 6-16, with the following amended paragraph:

(3) NPC fibroblasts were incubated with 1 μ M fPEG-Chol at room temperature for 5 minutes. Cells were then washed and incubated at 37°C for 30 minutes (Fig. ~~[[80]]~~ 40). NPC fibroblasts were incubated with 1 μ M fPEG-Chol at 4°C for 30 minutes. Cells were then washed and photographed. Cells were then washed and incubated at 37°C for 30 minutes (Fig. ~~[[8P]]~~ 4P). NPC fibroblasts were treated with 5 μ g/ml brefeldin A for 30

minutes (Fig. ~~[[8Q]]~~ 4Q), 5 μ g/ml nocodazole for 90 minutes (Fig. ~~[[8R]]~~ 4R), or 5 μ g/ml cytochalasin B for 30 minutes (Fig. ~~[[8S]]~~ 4S) before incubation with 1 μ M fPEG-Chol and 1 mg/ml rhodamine dextran for 30 minutes. In Fig. ~~[[8T]]~~ 4T, NPC fibroblasts were incubated with 1 μ M fPEG-Chol for 30 minutes before treatment with 5 μ g/ml cytochalasin B for 30 minutes. In Figs. ~~6 to 8~~ 4A-4T, the bar indicates 20 μ m.

Please replace the paragraph at page 17, line 18 to page 18, line 6, with the following amended paragraph:

Little has been known about the intra-membrane distribution of cholesterol. In the present example, whether or not cholesterol is located in the cytoplasmic side or luminal side of the intracellular membranes was examined by using semi-permeable cells. Plasma membranes of normal and NPC skin fibroblasts were selectively permeabilized by bacterial toxin streptolysin 0. Cells were then incubated with fPEG-Chol (Figs. ~~6A and 6B~~ 4A and 4B). The fPEG-Chol staining was dramatically different from those obtained in fixed and permeabilized cells (FIGS. ~~2A and 2E~~ to 2E). In addition, there was a big difference between normal and NPC cells. In normal skin fibroblasts, peripheral vesicle-like structures were strongly stained, whereas in NPC cells, ~~meshwork-like~~ meshwork-like structures were visualized. These structures were not observed after cells were fixed and permeabilized, suggesting that these compartments were either fragile or detergent sensitive. Golgi apparatus and late endosomes/lysosomes were not significantly labeled under these conditions. These results suggest that cholesterol resides only in the lumen of these organelles. In contrast, peripheral vesicles in normal fibroblasts and meshwork structures in NPC cells contain cholesterol in the cytoplasmic membranes.

Please replace the paragraph at page 19, lines 1 to 24, with the following amended paragraph:

The fate of cell surface fPEG-Chol of normal fibroblasts was compared with that of NPC fibroblasts (Figs. ~~7C to 7N~~ 4C to 4N). In the present experiment, 1 μ M fPEG-Chol was used. This concentration of fPEG-Chol did not affect the endocytosis of dextran and cholera toxin in this system. Cells were incubated with fPEG-Chol at room temperature for 5 minutes, washed, and further incubated at 37°C in the presence of 1 mg/ml rhodamine dextran. In normal fibroblasts, cell surface was strongly labeled after 5 minutes of fPEG-Chol labeling. Most of the fluorescence stayed on the plasma membrane after 10 minutes of chase (Figs. ~~7C and 7E~~ 4C and 4E). After 60 minutes of chase, nucleus became recognized as a non-labeled organelle surrounded by cytoplasmic fluorescent compartments (Fig. ~~[[7D]]~~ 4D). The overall pattern of these compartments was similar to that detected by DHE-M β CD in CHO cells (M. Hao et al., J Biol Chem 277, 609-17. (2002)). However, fPEG-Chol also stained intracellular vesicles. Most of these vesicles were not co-localized with internalized rhodamine dextran (Fig. ~~[[7G]]~~ 4G). These vesicles are often observed in the periphery of cells, like those observed in Fig. ~~[[6A]]~~ 4A. After 180 minutes, Golgi apparatus was prominently labeled with fPEG-Chol while rhodamine fluorescence was distributed in endosomes/lysosomes (Figs. ~~7E and 7H~~ 4E and 4H). The fate of fPEG-Chol was dramatically different in NPC fibroblasts. After 10 minutes of chase, fPEG-Chol stained characteristic meshwork structures (Figs. ~~7I and 7L~~ 4I and 4L), which was never observed in normal cells. Even after 180 minutes of chase, most of the fPEG-Chol was retained in this structure and Golgi fluorescence was hardly visible (Figs. ~~7J and 7M~~ 4J and 4M). Sometimes,

internalized rhodamine dextran was surrounded by the meshwork structures (Fig. [[7M]] 4M, arrows), suggesting that these structures have characteristics of endocytic compartments. These structures are very similar to those observed in Fig. [[6B]] 4B.

Please replace the paragraph at page 19, line 25 to page 20, line 12, with the following amended paragraph:

The incorporation of fPEG-Chol into the meshwork structure is temperature dependent. At 4°C, fPEG-Chol stayed on the plasma membrane and was not incorporated into the meshwork (Fig. [[8P]] 4P). Fig. [[8P]] 4P also indicates that fPEG-Chol does not undergo spontaneous transbilayer movement. The fluorophores, which undergo spontaneous flip-flop, stain intracellular membranes under these conditions (R. E. Pagano et al., J Cell Biol 91, 872-7. (1981); and R. E. Pagano et al., J Biol Chem 260, 1909-16. (1985)). Subsequently, the internalization of fPEG-Chol and rhodamine dextran was measured in the presence of inhibitors. Brefeldin A (an inhibitor of post-Golgi transport and nocodazole, which inhibits microtubule assembly) did not significantly affect the incorporation of fPEG-Chol into meshwork. In contrast, meshwork structure was disappeared by cytochalasin B (which inhibits actin polymerization). Cytochalasin B did not affect the internalization of rhodamine dextran. In Fig. [[8T]] 4T, cells were labeled with fPEG-Chol before treatment with cytochalasin B. In this case also, the meshwork structure was disappeared, suggesting that the meshwork structure is dependent on action network.